Corticotroph aggressive pituitary tumours and carcinomas frequently harbour ATRX mutations

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Abstract

Context: Aggressive pituitary tumours (APTs) are characterised by unusually rapid growth and lack of response to standard treatment. About 1-2% develop metastases being classified as pituitary carcinomas (PCs). For unknown reasons, the corticotroph tumours are overrepresented amongst APTs and PCs. Mutations in the ATRX gene, regulating chromatin remodelling and telomere maintenance, have been implicated in the development of several cancer types, including neuroendocrine tumours.

Objective: To study ATRX protein expression and mutational status of the ATRX gene in APTs and PCs.

Design: We investigated ATRX protein expression by using immunohistochemistry in 30 APTs and 18 PCs, mostly of Pit-1 and T-Pit cell lineage. In tumours lacking ATRX immunolabeling, mutational status of the ATRX gene was explored.

Results: Nine of the 48 tumours (19%) demonstrated lack of ATRX immunolabelling with a higher proportion in patients with PCs (5/18 - 28%) than in those with APTs (4/30 – 13%). Lack of ATRX was most common in the corticotroph tumours, 7/22 (32%), vs 2/24 (8%) in the tumours of the Pit-1 lineage. Loss-of-function ATRX mutations were found in all the nine ATRX immuno-negative cases: nonsense mutations (n=4), frameshift deletions (n=4) and large deletions affecting 22-28 of the 36 exons (n=3). More than one ATRX gene defect was identified in two PCs.

Conclusion: ATRX mutations occur in a subset of aggressive pituitary tumours and are more common in corticotroph tumours. The findings provide a rationale for performing ATRX immunohistochemistry to identify patients at risk of developing aggressive and potentially metastatic pituitary tumours.

Keywords: ATRX (alpha thalassemia/mental retardation syndrome X-linked); aggressive PitNETs; pituitary carcinoma; pituitary adenoma; Cushing's disease.

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Introduction

Pituitary neuroendocrine tumours (PitNETs) (1), traditionally designated as pituitary adenomas, are usually benign tumours with indolent, non-aggressive course. Recently, the European Society of Endocrinology published criteria that define aggressive PitNETs as tumours demonstrating an unusually fast growth and/or lack of response to all standard treatment modalities including surgery, radio- and pharmacological therapies (2). Pituitary carcinomas (PC) are defined by the presence of non-contiguous craniospinal or distant metastases (3). While PC are rare and comprise only 0.1-0.2% of all pituitary neoplasms (4), the prevalence of aggressive pituitary tumours (APT) without metastases is less well known. An estimate of 3% has been suggested based on indices of increased proliferation and extensive p53 staining in tumour specimens from 451 patients reported to the German Pituitary Tumour Registry (5). Little is known about genetic abnormalities driving invasive and metastatic pituitary tumours. Whether they develop through malignant progression of benign pituitary tumours or occur as de novo malignant tumours caused by early, single or multiple genetic changes predisposing for distant dissemination is unknown.

The functioning corticotroph tumours causing Cushing´s disease represent less than 5% of the benign, slow-growing PitNETs (6,7). However, they are overrepresented amongst APT and PC where they constitute approximately 30-40% (8,9). One suggested explanation for this was a lower expression of the cell cycle inhibitor p27 in normal corticotroph cells and corticotroph tumours (10); however, the mechanisms are still unclear. Silent corticotroph tumours are also considered potentially more aggressive according to the current World Health Organisation (WHO) classification of the pituitary tumours (3), although a recent meta-analysis could not identify an increased recurrence rate in this subtype (11).

In patients with aggressive pituitary tumours, genetic abnormalities have previously only been reported in single sporadic cases, none has consistently been found in larger groups of

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patients (12). In a case of clinically non-functioning gonadotroph carcinoma, a low level of HER2/neu gene amplification was demonstrated by using FISH and CISH analysis (13). The presence of mi-RNAs probably targeting PTEN (phosphatase and tensin homolog) and TIMP2 (tissue inhibitor of metalloproteinases 2) was reported as potential drivers of metastatic growth in a case with a non-functioning PC (14). A single case of PC was reported in a patient with succinate dehydrogenase subunit B gene mutation and history of paraganglioma (15). Finally, tumour protein p53 mutations in two PC have been described (16).

ATRX interacts with death domain-associated protein (DAXX) and the histone H3.3 variant in heterochromatin remodelling and maintenance of telomere structure and function (17,18). Inactivation of ATRX or, less frequently DAXX in ATRX/DAXX mutated tumours, leads to telomere destabilisation and facilitates the process of alternative lengthening of telomeres (ALT), which results in cancer cell immortality (19,20). Somatic ATRX gene mutations are associated with several different tumour types, including astrocytomas in adults (21) and neuroendocrine tumours (NET) such as pancreatic NETs (22,23), neuroblastomas (24) and paragangliomas/pheochromocytomas (25,26). Interestingly, in neuroendocrine tumours, ATRX abnormalities seem to predict malignant tumour phenotype, being present in high grade malignant tumours such as neuroblastoma (24), or associated with poor prognosis and/or metastatic potential, such as in pancreatic NET (27), and pheochromocytomas/paraganglioma (26).

We have previously demonstrated normal immunohistochemical expression of ATRX protein in a large cohort of 246 well-characterised PitNETs localised to the sellar region, including 37 corticotroph tumours. However, one of two studied pituitary carcinomas (a corticotroph carcinoma in a patient with Cushing´s disease) did not express the protein due to a large deletion of the ATRX gene (28).

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In the present study, we aimed to further explore ATRX protein expression and mutational status of the ATRX gene in a large cohort of aggressive PitNETs and pituitary carcinomas.

Material and methods

Patient cohort

Pituitary tumour specimens were obtained from a multicentre cohort of 48 patients (15 F, 33 M), with a median age 45 (range 16-73 years) at diagnosis. Inclusion criteria were at least one pituitary surgery and tumour progression despite radiotherapy, and/or while on treatment with dopamine agonists or somatostatin analogues, or metastatic disease. Thirty patients had APT and 18 had PC with cerebrospinal and/or systemic metastases. The median time from diagnosis of the pituitary tumour to metastases was 8.5 (range 1.2 - 36) years (Table 1). The patients were treated at specialised centres in 11 European countries (Belgium, Denmark, Finland, France, Hungary, Italy, Norway, Poland, Serbia, Sweden and UK). Patients' data and tumour characteristics at the first presentation, treatments given, and outcome were collected in anonymised standardised questionnaires filled in by all participating centres.

Information on pituitary tumour size and local extension at the first magnetic resonance imaging (MRI) was available in 45 and 43 patients, respectively. All but one lactotroph tumour were macroadenomas at the time of diagnosis. By the time of pituitary surgery, invasion of the cavernous sinuses, bone and/or brain was evident on MRI in the 39 cases, including the single patient who had a microadenoma. Of the 48 patients, 39 had more than one pituitary surgery, and 33 more than two. Forty-six out of the 48 patients had received at least one radiotherapy. In one case, tumour size and extension were considered too large for radiotherapy, and in the second case, the reason for not performing radiotherapy was not available. No tumour treated with dopamine agonists and/or somatostatin analogues (octreotide, lanreotide, pasireotide) was controlled by these medications (Table 1). In addition to standardised medical therapy, 34 patients had received treatment with chemotherapy, temozolomide in 33 including one patient with additional bevacizumab, and another one with an mTOR inhibitor and two immune checkpoint inhibitors.

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Tumours were classified based on the laboratory and clinical signs of pituitary hormone hypersecretion, expression of anterior pituitary hormones in the tumour cells, and, in the cases of hormone-negative non-functioning tumours, by their expression of pituitary specific transcription factors. Corticotroph tumours were the most common, 22/48, of which 16 were functioning tumours causing Cushing's disease. Lactotroph tumours were the second most common, n=15 (Table 1).

The index patient with ATRX mutation has been previously reported (28) and is also included in the present study. Of the 48 patients, three had syndromes predisposing for pituitary tumours, one had MEN1 (29), one had Lynch syndrome (30), and one patient belonged to a kindred with familial predisposition for pituitary tumours (FIPA), however, without MEN1 or AIP mutation. In addition, pituitary tumour tissue from a corticotroph non-aggressive macroadenoma in a patient with Lynch syndrome was investigated. This case was not included in the statistical analyses as it did not fulfil criteria for aggressive tumours.

In 45 patients, at least one specimen from pituitary surgery was available for analyses. In the remaining three patients, there was only specimen from the metastasis. For seven patients with carcinoma, material from both pituitary surgery and from metastatic tumour was available. The presence of representative tumour tissue was confirmed in haematoxylin-eosin stained slides from all specimens.

Immunohistochemical analyses

Immunohistochemistry (IHC) with antibodies towards growth hormone (GH), prolactin (PRL), thyrotroph hormone (TSH), adrenocorticotroph hormone (ACTH), gonadotroph hormones, follicle-stimulating hormone (FSH) and luteinising hormone (LH), was performed at the local IHC laboratories according to the routine protocols. Immunohistochemical analysis with antibodies towards pituitary-specific transcription factors was performed at Uppsala University Hospital by using anti-SF1 antibody (Abcam, ab217317), anti-Pit-1 antibody (Novus Biologicals, NBP1-92273), and anti-T-Pit antibody (Atlas Antibodies, AMAb91409), according to the standard protocols.

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ATRX protein expression was studied on whole sections from formalin fixed paraffin embedded tissue blocks. For the patients operated more than once, available tissue specimens from multiple surgeries were examined. In the majority of cases, IHC was performed at Uppsala University Hospital in a DAKO-Autostainer Link 48 with heat-induced epitope retrieval at high pH. Purified polyclonal anti-ATRX antibody (HPA001906, Atlas Antibodies; dilution 1:100; incubation time 20 min) was used. Specimens from two adult astrocytomas, one with ATRX mutation and one without ATRX mutation, both confirmed by using molecular genetic analysis, were used as negative and positive controls. In addition, immunolabelled endothelial cells served as an internal positive control. Four cases from Foch Hospital (Suresnes, France) and a case from University Hospital in Copenhagen, Denmark were stained in Ventana Benchmark by using the same antibody and according to the locally optimised protocols.

Molecular genetic analysis

Molecular genetic analysis was performed on tumour tissue from the pituitary specimen in all nine cases demonstrating lack of ATRX immunolabelling. In two patients, specimens from metastases were also analysed. If there was more than one specimen from the pituitary surgery, the specimen with the most representative tumour tissue was used. In one patient, a partial lack of ATRX protein labelling was observed in the pituitary specimen and a total lack in metastatic tumour tissue. In this patient, an attempt was made to microdissect tissue and extract DNA separately from ATRX negative and positive area of the pituitary tumour. In addition, the specimen from metastasis with negative ATRX staining was analysed.

All but one specimen were examined by a next generation sequencing panel targeting 20 genes (31) related to cancers of the central nervous system as in the initial study (28). Proportion of tumour cells exceeded 70% in all the specimens. One specimen was analysed using an exome-wide sequencing approach.

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Next generation sequencing (NGS)

DNA was purified from 10 µm paraffin slides using GeneRead DNA FFPE Kit (Qiagen, Germany) according to the manufacturer's instructions. NGS was performed with a custom designed central nervous system (CNS) panel covering the entire coding sequence or hotspot regions of 20 genes frequently mutated in brain tumors (32). DNA was quantified using an RNase P TaqMan Copy Number Reference Assay performed on a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). Libraries were prepared in two primer pools using the Ion AmpliSeq Library Kit Plus and Ion Xpress Barcode Adapters 1–96 Kit in 10 µL reaction volume with 5 ng template DNA. Library quantitation was performed using the Ion Library Quantitation Kit. Sample preparation, chip loading and sequencing were performed using Ion Chef and Ion Torrent S5 System with Ion S5 Chef solutions, Ion S5 sequencing reagents and Ion 530/540 Chip Kits. All Ion products were supplied by Ion Torrent/ThermoFisher Scientific, Carlsbad, CA, USA. Data analysis, including base calling, quality scoring, trimming, demultiplexing, and alignment, was performed using standard Ion Torrent Suite v5.10 workflows. BAM alignment files were manually analysed for alterations in the coding sequences of the twenty genes using Golden Helix GenomeBrowse 3.0 (Golden Helix, Bozeman, MT, USA). The sequencing experiments included ATRX wild-type control samples from healthy donors.

One specimen was analysed using hybridization capture-based high-throughput NGS platform from Illumina (33).

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Ethical approval

The study has been approved by Regional Ethical Committee in Uppsala (Dnr 2018/327).

Results

Lack of ATRX protein expression is frequent in corticotroph tumours

Nine of the 48 tumours (19%) demonstrated lack of ATRX immunolabelling in the tumour cells. Five were carcinomas and seven were corticotroph tumours, representing 32% of all corticotroph tumours (7 out of 22). Lack of protein expression was more common in patients with functioning corticotroph tumours, 6/16 (38%) than in those with silent corticotroph tumours (1/6, 17%). Of the remaining two ATRX-immunonegative tumours, one was a lactotroph APT with a fatal outcome, and one was a somato-lactotroph carcinoma that initially presented as a prolactinoma and subsequently evolved into acromegaly (Table 2).

More than one pituitary specimen was available for analysis in six of seven patients who underwent multiple surgeries. In five of the six patients, all specimens demonstrated lack of ATRX in all tumour cells. In one patient, the specimen from the first surgery could not be assessed, there was partial lack of ATRX expression in pituitary tumour from the second surgery and a total lack in the metastasis. In five patients with PC, specimens from metastases were available in four and demonstrated negative ATRX staining in the tumour cells. The remaining 39 pituitary tumours demonstrated intact nuclear ATRX expression.

Examples of PitNETs with normal ATRX staining, total lack of immunolabelling and partial negative ATRX staining in primary and metastatic tumours are illustrated in Figure 1.

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All ATRX-immunonegative tumours harbour loss-of-function ATRX gene abnormalities

ATRX loss-of-function gene abnormalities were found in all nine ATRX-immunonegative tumours (Table 3) (31). Two different, damaging ATRX mutations with large differences in mutation frequencies were identified in the same primary tumour in two carcinomas from male patients. One of these two tumours demonstrated a partial lack of ATRX at IHC. An attempt to extract separately DNA from ATRX-immunopositive and negative fraction was, however, unsuccessful, as the same mutational status was confirmed in both fractions. Interestingly, only the predominant mutation from this pituitary tumour was present in the metastasis (six years later) with a frequency of 98%, suggesting clonal heterogeneity and evolution of the primary tumour (Table 3) (31). Three tumours did not show any ATRX single nucleotide variants (SNVs) or small indels, but had large, intragenic deletions corresponding to most of the coding sequences (22-28 of 36 exons) (Fig. 2A, B). One of these tumours was the corticotroph tumour previously reported, whereas the other two were lactotroph and somato-lactotroph, respectively. All identified ATRX SNVs and small indels were positioned throughout the coding sequence of the ATRX gene (Fig. 2C).

In addition to the ATRX mutations, eight out of nine ATRX-immunonegative tumours had other genetic abnormalities: inactivating somatic mutations in tumour suppressor genes TP53 (six), PTEN (two), RB1 (one), NF2 (one), and a homozygous deletion of CDKN2A/B in both primary tumour and metastasis in one patient (Table 3). Recurrent CNVs that were estimated from the sequencing data were all gains, and involved chromosomes 5, 7, 9p21.3 encompassing CDKN2A/B loci as well as the CIC locus on 19q.

Discussion

Little is known about genetic abnormalities driving invasive and metastatic growth of PitNETs. Here, we demonstrate a loss of ATRX protein expression caused by severe loss-of-function ATRX gene alterations in almost a fifth of highly aggressive pituitary tumours, with a higher prevalence in PC compared to APT, and in corticotroph tumours compared to other lineage subtypes. This indicates that corticotroph tumours are prone to develop ATRX gene abnormalities.

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We reported previously normal ATRX expression in 246 PitNETs localised to the sellar region. However, in one female patient diagnosed with Cushing's disease and a pituitary macroadenoma at an age of 36 years, we found negative ATRX immunolabelling caused by a large deletion of the ATRX gene (28). This tumour had progressed over time and had become metastatic despite multiple transsphenoidal surgeries, pharmacological therapy, and three different modalities of radiation therapy. ATRX staining was absent in all the tumour specimens including the one from the first surgery.

In the present extended study, we demonstrate ATRX gene defects in eight additional patients. Thus, nine out of 48 patients (19%) with aggressive pituitary tumours or carcinomas harboured loss-of-function ATRX gene alterations, more frequently in patients with PC compared to APT (28% vs 13%). Five out of the total nine patients with ATRX gene defects had carcinomas. Of the four APT patients, two died due to progressive tumour growth, in another there was a short time from the tumour diagnosis to the study end, and in the last patient, search of metastases was not performed due to advanced dementia. Further studies with longer follow-up are needed to assess to what extent an initial ATRX defect leads to a metastatic disease.

In addition to our previously reported case of ATRX mutated corticotroph carcinoma (28), a corticotroph carcinoma with an ATRX mutation in combination with PTEN and TP53 mutations has been described; however, without detailed presentation of genetic data (34).

In a recent study (35), whole exome sequencing of 18 corticotroph tumours lacking mutations in USP8 (ubiquitine specific peptidase 8) gene, mutations that drive corticotroph tumours in approximately 50 % of patients with Cushing's disease, demonstrated ATRX mutations concomitantly with TP53 mutations in two. Although detailed clinical data regarding aggressiveness of the two ATRX mutated tumours were not presented, both were recurrent and required surgery on two and >3 occasions, respectively, and Ki67 proliferative index was increased in one of the cases

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(35). Lack of ATRX immunolabelling was recently found in three lactotroph macroadenomas from a cohort of 42 paediatric PitNETs, but molecular genetic confirmation of the ATRX mutations was not provided (36). Recently, ALT phenotype has been reported in three of 106 PitNETs, two were recurrent non-functioning PitNETs without specification of cell-linage differentiation, and one was a somatotroph tumour (37). Two of the three ALT-positive PitNETs demonstrated loss of ATRX or DAXX at protein level indicating a homozygous loss of the gene or alternative mechanism of gene silencing. However, no ATRX or DAXX mutations were identified by sequencing (37).

In patients who had repeated pituitary surgeries in the present cohort, an ATRX defect was already present in the first removed tumour, though, in one patient, tumour tissue from the first surgery was not evaluable. This indicates that ATRX abnormalities represent an early genetic event contributing to aggressive behaviour and, at least in a subset of patients, to metastatic spread. Where material from both the pituitary tumour and metastasis was available (n=4), identical patterns of a complete loss of ATRX were seen in three, whereas in one case, partial loss of ATRX was identified in the pituitary tumour and a complete loss in the metastasis. A similar case of a PitNET with ALT-negative phenotype in the original tumour, and ALT-positive phenotype and a partial loss of ATRX in a recurrent tumour, was recently reported (37). These findings suggest that an ATRX mutation may occur, though rarely, in pituitary tumours with primarily intact ATRX, contributing to malignant tumour progression.

In the ATRX-mutated cases in our cohort, we demonstrated different loss-of-function ATRX defects including nonsense mutations, frameshift indels and, in three cases, large, intragenic deletions of almost the whole gene (22 to 28 of the 36 exons). Interestingly, large deletions of almost the whole ATRX gene have only rarely been reported in other tumour types, such as astrocytomas (21,32), pancreatic NETs (22) and pheochromocytomas and paragangliomas (25). Yet, a recent study on ATRX alterations in neuroblastoma demonstrated a strong tendency for large, intragenic deletions of exons 1-9, encoding the first half portion of the ATRX protein (38). In our cohort, there was no predominance of a particular type of mutations in carcinomas compared to APTs, or in corticotroph

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compared to Pit-1-lineage tumours. However, the number of mutated cases may be too low to make conclusions on a potential genotype-phenotype association.

Blood samples or normal tissues from patients were not included in the sequencing experiments to test for germline mutations. The variant allele frequencies (VAFs) of mutations in ATRX reported in this study are in favour of somatic rather than germline origin. Furthermore, IHC revealed normal ATRX expression in non-neoplastic cells in all the mutated specimens, arguing for the somatic origin of the ATRX gene defects.

In the present study, we had the opportunity to investigate ATRX in two patients with corticotroph tumours, one non-aggressive macroadenoma and one carcinoma, and Lynch syndrome, a cancer predisposing syndrome with mutations in genes involved in DNA mismatch repair (MLH1, MSH2, MSH6, PMS2, EPCAM). Both tumours harboured an MSH2 mutation, but only the severe case, a carcinoma, in addition exhibited an ATRX mutation.

Additional cancer-related mutations were identified and associated with ATRX alterations in eight of nine cases, TP53 mutations in six (three aggressive corticotroph tumour, two corticotroph carcinomas and one aggressive lactotroph tumour), PTEN mutations in two, and RB1, NF2 and CDKN2A/B in single cases. TP53 mutations have rarely been previously reported in pituitary tumours (16). However, recently, TP53 mutations were demonstrated in six out of 18 of corticotroph USP8 wild-type tumours and correlated with larger tumours and higher Ki67 index (35). Our findings, together with previous report, may suggest an association of the TP53 mutations with corticotroph tumours with more aggressive phenotype. Findings of multiple mutations in the ATRX mutated tumours may indicate genetic instability leading to multiple cancer-related genetic events. However, more extensive

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molecular genetic analyses are needed to get full insight into genetic landscape of aggressive PitNETs.

The strength of the present study is the well characterised cohort of APT and PC and a relatively large number of patients, having in mind the rarity of the condition. A limitation is a short follow up of some of the patients with ATRX defects, which limits conclusions on the metastatic potential of this mutation.

Although many APT/carcinomas exhibit histological features consistent with increased proliferation (Ki-67 index > 3%, increased mitotic count, and p53 expression) (4), and co-existence of two of the three markers is associated with increased risk of tumour progression and recurrence (39), the presence of these features does not fully predict future aggressive behaviour (40,41). To our knowledge, the present findings is the first time that a gene mutation with well-known oncogenic potential has been consistently reported in a proportion of aggressive PitNETs.

Currently, temozolomide is the first line chemotherapy for APT and PC (29). The drug induces an initial response rate of 40%, but subsequently most tumours relapse and long-term effective alternative therapies are still lacking (42). Mutated ATRX is an attractive therapeutic target for the subgroup of ATRX negative pituitary tumours. There is ongoing intensive research aiming to develop pharmacological therapies targeting ATRX and ALT (43,44).

In summary, the results of this study provide a rationale for performing ATRX immunohistochemistry as a simple, inexpensive and widely available laboratory test to identify patients at increased risk for development of highly aggressive and potentially metastatic PitNETs, especially in macroadenomas causing Cushing's disease or in clinically silent corticotroph tumours. Patients with pituitary tumours harbouring an ATRX mutation should be offered closer follow-up, including work-up for metastatic dissemination, and invasive treatment at the early stages of the disease.

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Data Availability: Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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Figure legends:

Table 1. Patient and tumour characteristics in the study population.

Table 2. Patient and tumour characteristics in ATRX mutated vs intact cases.

Table 3. Genetic alterations in ATRX-immunonegative APT and PC by panel NGS.

Figure 1. Histopathological and immunohistochemical features of PitNETs. Row 1: Haematoxylin eosin staining of a primary pituitary tumour invading into the respiratory mucosa (1A) with a total lack of ATRX in tumour cells nuclei both the primary pituitary tumour (1B) and a lymph gland metastasis (1C) in a patient with a functioning somato-lactotroph carcinoma. ATRX expression is intact in respiratory epithelium, endothelial cells and lymphocytes. Row 2: Haematoxylin eosin staining from the primary pituitary tumour in a patient with a silent corticotroph carcinoma (2A). A partial nuclear ATRX-loss in a proportion of cells in the specimen from the second pituitary surgery (2B) and a total ATRX-loss in the metastasis (2C) ATRX expression is preserved in the nuclei of the endothelial cells. Row 3: Haematoxylin eosin staining of the specimen from the first surgery (3A) and normal ATRX expression in the nuclei of the tumour cells in the specimens from two pituitary surgeries (3B, 3C) in a patient with a silent Pit-1 positive PitNET.

Figure 2. ATRX deletions in two patients with corticotroph carcinoma. A. Profile of the average amplicon coverage of ATRX coding sequence from exon 1 through exon 35. A healthy donor of female origin was included in all NGS experiments (blue). Low amplicon coverage corresponding to deletion of ATRX sequence was observed for two patients, highlighted with orange and green, respectively. Coverage overviews are shown as inserts. The black horizontal bar indicates the position of chr. X. Arrows mark the deletions in ATRX. B. Schematic illustration of the two large, intragenic ATRX deletions spanning exon 2/exon 8 through exon 30. The large exon 9 of ATRX, depicted with stippled lines, is compressed for clarity. C. Diagram of ATRX variants at coding and protein levels, respectively. Definition of ATRX domains were from UniProt. ADD: ATRX-DNMT3-DNMT3L. Helicase C: Helicase C-terminal.

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Table 1. Patient and tumour characteristics in the study population

	Total	APT	PC
Total n	48	30	18
Age at diagnosis, yr (median, range)	45 (16-73)	46.5 (18-73)	42 (16-69)
Male n (%)	33 (69)	23 (77)	10 (56)
Macroadenomas[a]	44/45	28/29	16/16
Invasive growth[a]	39/42	24/27	15/15
No of surgeries (median, range)	3 (1-10)	3 (1-10)	3.5 (1-8)
No of radiotherapies (median, range)	1 (0-4)	1 (0-2)	2 (1-4)
Resistance to DA/ somatostatin analogues[b]	27/27	18/18	10/10
Time to metastases from 1st surgery, yr (median, range)			8.5 (1.2-36)
Treatment with cytotoxic drugs[b]	35/37	21/23	14/14
ATRX negative, n (%)	9 (19)	4 (13)	5 (28)
Tumour subtypes (IHC)			
- Corticotroph[c]	22	10	12
- Lactotroph	15	12	3
- Somatotroph	4	2	2
- Somato/lactotroph	2	1	1
- TSH/FSH	1	1	0
- Silent Pit 1 positive PitNET	3	3	0
- Null cell	1	1	0

APT, aggressive pituitary tumours; PC, pituitary carcinoma; DA, dopamine agonists; IHC, immunohistochemistry; [a]MRI at first tumour presentation in patients with available information. [b]in patents with available information. [c]Six clinically silent (2 PC, 4 APT).

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Table 2. Patient and tumour characteristics in ATRX mutated vs intact cases

	ATRX mutated	ATRX intact
Total n	9	39
Age at diagnosis, yr (median, range)	45 (23-72)	45 (16-73)
Male, n (%)	6 (67)	27 (69)
Aggressive pituitary tumours, n (%)	4 (44)	26 (67)
Pituitary carcinomas, n (%)	5 (56)	13 (33)
Tumour subtypes (IHC)		
- Corticotroph (n=22)	7	15
PC (n=12)	4	8
APT (n=10)	3	7
- Lactotroph (n=15)	1	14
PC (n=3)	0	3
APT (n=12)	1	11
- Somato/lactotroph (n=2)	1	1
PC (n=1)	1	0
APT (n=1)	0	1
- Other subtypes[a] (n=9)	0	9
PC (n=2)	0	2
APT (n=7)	0	7

IHC, immunohistochemistry; [a]somatotrophs (4); silent Pit 1 positive (3); double TSH/FSH (1); null cell PitNET (1)

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Table 3. Genetic alterations in ATRX-negative APT and PC by panel NGS

Pt.	Specimen	Local.	ATRX expression	Genes	Coding	Amino Acid	Freq. (%)#
1	Cushing/PC	Pituitary	loss	*ATRX*	c.134_6217del	p.D45-K2027del	Nu
2	Cushing/PC	Pituitary	loss	*ATRX*	c.748C>T	p.Arg250Ter	89
	Lynch sy			*TP53*	c.524G>A	p.Arg175His	84
				PTEN	c.697C>T	p.Arg233Ter	10
3	Lactotroph/ APT	Pituitary	loss	*ATRX*	c.21_6699del	p.E8-K2233del	Nu
				TP53	c.584T>A	p.Ile195Asn	92
				RB1	c.1725_1726 insAACAA	p.Ser576fs	13
				RB1	c.1218_1697del	p.N406-S565del	He
4	Cushing/PC	Pituitary	loss	*ATRX*	c.6679delG	p.Asp2227fs	81
				ATRX	c.3583delA	p.Arg1195fs	12
5*	Silent ACTH/PC	Pituitary	retained (major)/ loss (minor)	*ATRX*	c.4048_4049del GG	p.Gly1350fs	28
				ATRX	c.6661G>T	p.Glu2221Ter	31
				TP53	c.644G>A	p.Ser215Asn	30
5*	Silent ACTH/PC	Pituitary	loss (major)/ retained (minor)	*ATRX*	c.4048_4049del GG	p.Gly1350fs	67
				ATRX	c.6661G>T	p.Glu2221Ter	10
				TP53	c.644G>A	p.Ser215Asn	8
5	Silent ACTH/PC	Metastasis	loss	*ATRX*	c.4048_4049del GG	p.Gly1350fs	98
6	Cushing/APT	Pituitary	loss	*ATRX*	c.2422C>T	p.Arg808Ter	72
				TP53	c.1024C>T	p.Arg342Ter	51
				PTEN	c.697C>T	p.Arg233Ter	55
7	Cushing/APT	Pituitary	loss	*ATRX*	c.839_840insCATG	p.Asn281Ter	44
				TP53	c.818G>A	p.Arg273His	85
				NF2	c.1052G>A	p.Arg351His	20
8	Cushing/APT	Pituitary	loss	*ATRX*	c.5938T>A, c.5939delC	p.Ser1980fs	88
				TP53	c.375G>A	p.(=)	81
9	Mixed GH-PRL/PC	Pituitary	loss	*ATRX*	c.595_6699del	p.N199-K2233del	He

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				CDKN2A	c.1_501del	p.M1-A167del	Ho
				CDKN2B	c.1_414del	p.M1-D138del	Ho
9	Mixed GH-PRL/PC	Metastasis	loss	*ATRX*	c.595_6699del	p.N199-K2233del	He
				CDKN2A	c.1_501del	p.M1-A167del	Ho
				CDKN2B	c.1_414del	p.M1-D138del	Ho

APT, aggressive pituitary tumours; PC, pituitary carcinoma; NGS, Next-generation sequencing; ACTH, Adrenocorticotropic hormone, GH, growth hormone; PRL, prolactin; #, Estimated ploidy level of larger gene deletions: Nu: nullizygous, He: hemizygous deletion, Ho: homozygous deletion; * The same mutations were detected in ATRX immunopositive and immunonegative tissue fractions indicating that they could not be successfully separated.

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Figure 1

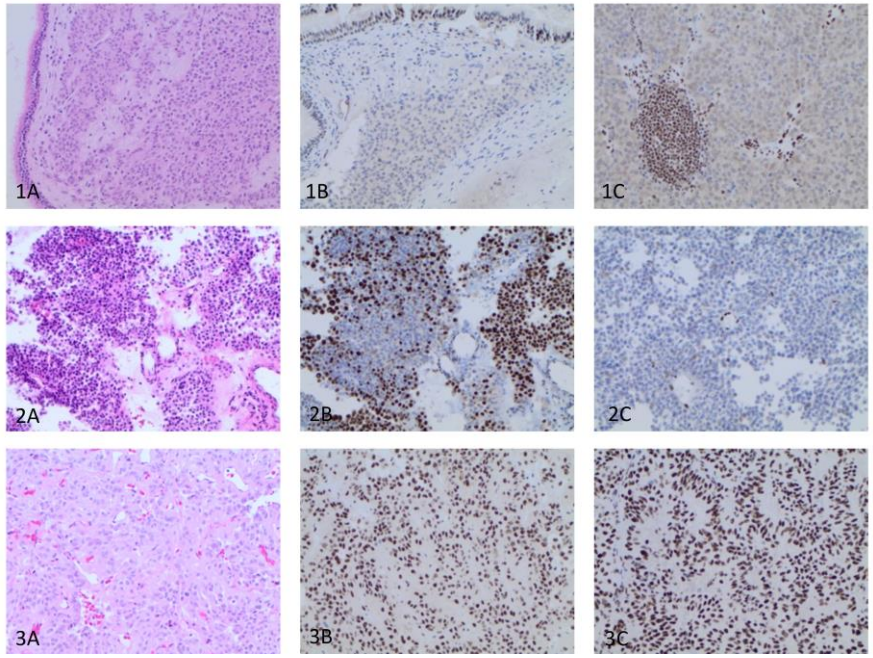

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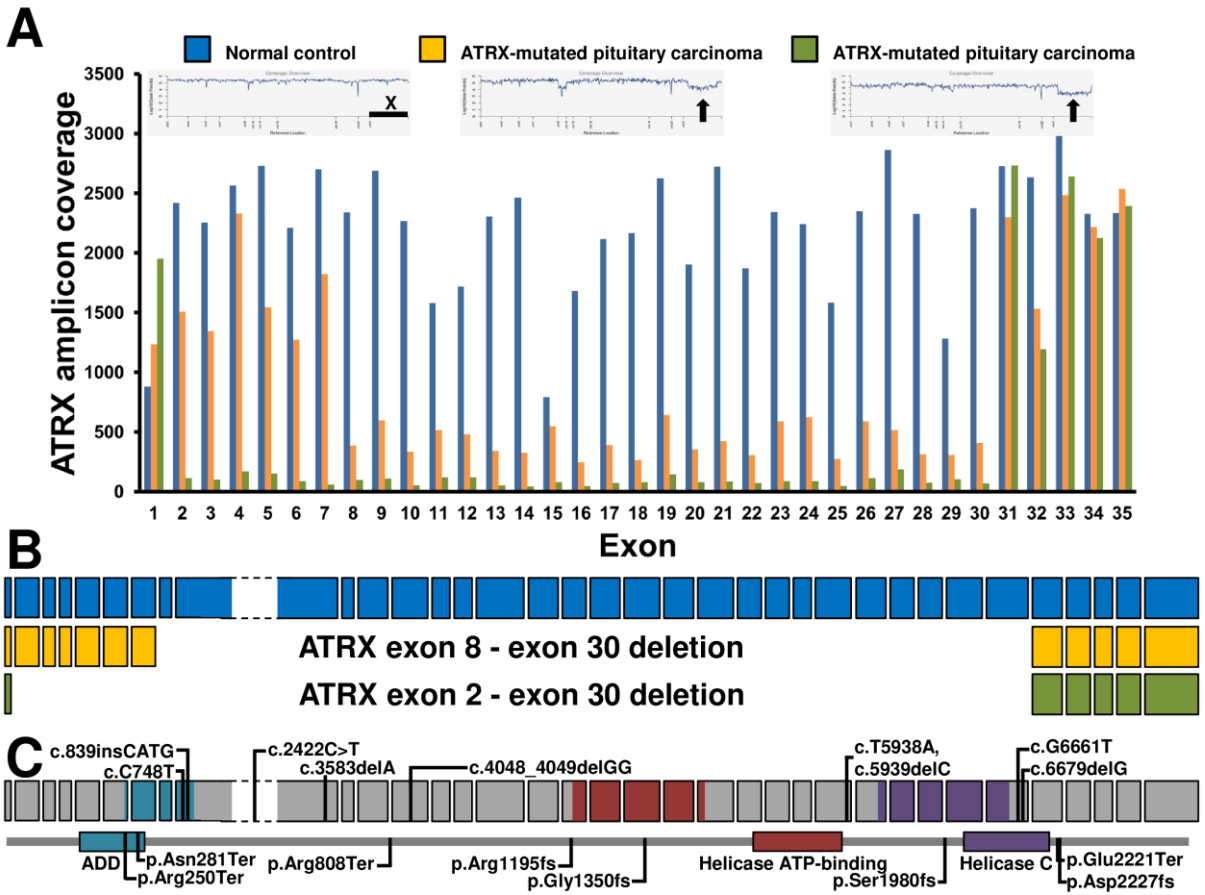
Figure 2



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